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<b>(21) International Application Number:</b> PCT/DK97/00317 <b>(22) International Filing Date:</b> 23 July 1997 (23.07.97) <b>(30) Priority Data:</b> 8/208422                      7 August 1996 (07.08.96)                      JP <b>(71) Applicant (for all designated States except US):</b> NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsvaerd (DK). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> MIYOTA, Yoshiaki [JP/JP]; Showa Denko K.K., Central Research Laboratory, 1-1, Ohnodai 1-chome, Midori-ku, Chiba-shi, Chiba 267 (JP). FUKUYAMA, Shiro [JP/JP]; Showa Denko K.K., Central Research Laboratory, 1-1, Ohnodai 1-chome, Midori-ku, Chiba-shi, Chiba 267 (JP). <b>(74) Common Representative:</b> NOVO NORDISK A/S; Corporate Patents, Novo Allé, DK-2880 Bagsvaerd (DK).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> DOUBLE-STRANDED DNA WITH COHESIVE END(S), AND METHOD OF SHUFFLING DNA USING THE SAME <b>(57) Abstract</b> <p>To provide a method of mutation of DNAs, which is substantially different from the conventional methods applicable to naturally-existing DNAs, and also to provide useful genetic products to be produced by the use of thus-mutated DNAs. A DNA with a cohesive end comprising (a) a double-stranded DNA having the same sequence as that of a part of a gene, and (b) a single-stranded DNA having a base sequence that exists on said gene at the site not adjoining the part corresponding to said double-stranded DNA or a base sequence which said gene does not have, wherein the single-stranded DNA is linked to either one end of the double-stranded DNA to form a cohesive end; a method for producing it; a method of shuffling a DNA using it; a DNA and a DNA pool to be obtained by the shuffling method; a method for producing the DNA pool; and a genetic product to be obtained by expressing the genetic information existing in the DNA pool.</p>		

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**Title: Double-stranded DNA with cohesive end(s), and method of shuffling DNA using the same**

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#### **INDUSTRIAL FIELD**

The present invention relates to a double-stranded DNA with a cohesive end or cohesive ends having a desired sequence and a method for producing it, and also a method for shuffling  
10 a DNA using DNA blocks with a cohesive end or cohesive ends, the DNA as shuffled according to the method, a DNA pool to be obtained according to the shuffling method, and also a genetic product to be produced by the use of the DNA pool.

#### **15 BACKGROUND ART**

One approach to protein engineering for improving naturally-existing proteins to modified ones which are more useful to human beings is to improve proteins through site-specific mutation, which has produced some results (Japanese Patent  
20 Application Laid-Open No. 5-91876). However, this requires the clarification or identification of the stereostructure of the targeted protein, and much labor is needed for the analysis of the stereostructure. In addition, even though the stereostructure could be clarified or identified, there are still many  
25 unknown matters for the relationship between the structure and the function with proteins. Therefore, it is still difficult to surely impart an intended function to the targeted protein.

In order to overcome these difficulties, a process comprising random mutation and screening and also evolutionary molecular engineering that utilizes the evolution of organisms have been being highlighted and said to be extremely useful (Proc. Natl. Acad. Sci., USA, 83, 576 (1986)). However, the current methods are directed to the substitution of at most several amino acids.

In WO95/22625, disclosed is a method for forming novel genes by dividing a plurality of genes at random and homologically recombining them to reconstruct novel genes. However, this is one method for forming chimera genes. The genes to be formed by this method are similar to the original genes, and the former shall have the essential base sequences of the latter.

Using such known methods, it is difficult to desire the impartation of some additional functions to organisms which they could not gain during the steps of their evolution. In order to obtain genetic products, of which the functions are greatly different from those of naturally-existing substances such as proteins, it is believed effective to prepare a pool of nucleic acids having significantly different base sequence spaces from those existing naturally, and to produce from them genetic products having the intended functions.

One method for this may be to prepare a nucleic acid pool that covers all base combinations. However, even the total number of the base sequences that may code for a relatively small protein with 100 amino acids (300 bp) is an enormous

number of  $4^{300}$  (about  $10^{180}$ ), and it is in fact impossible to prepare the nucleic acid pool that may cover all of them.

For proteins of some kinds, their sub-structures which are referred to as modules were specifically noted, and an attempt was made to change the sequencing of the base sequence blocks corresponding to the individual modules to thereby produce mutants having different module sequences (Viva Origino, Vol. 23, No. 1 (1995) 86-87). In this attempt, however, the base sequences were re-sequenced merely individually for the individual mutants. No one has heretofore attempted the formation of a nucleic acid pool covering all re-sequenced molecules and the collection of genes capable of expressing products having intended properties from the pool.

Utilizing restriction enzymes, it is possible to prepare a nucleic acid pool including various molecules by blending several kinds of DNA blocks having the same cohesive end or blunt end followed by ligating them at random, and to select from this pool some molecules having desired properties. According to this method, however, the DNAs to be used must have predetermined restriction enzyme recognizing sites. Even though the DNAs have such restriction enzyme recognizing sites, there is an extremely small probability that the sites are positioned at the desired sites. In this method, in addition, the both ends of the blocks must be of the same type, and there is a high probability that the blocks are therefore self-ligated. A means of forming the restriction enzyme recognizing sites through site-specific mutation may be taken in order to overcome these problems. However, the matter as to whether or not

the blocks can be ligated in accordance with the predetermined frame is likely much governed by chance. That is, the matter as to whether or not a desired protein can be produced without misreading the reading frame of the codon shall be governed by  
5 chance. Therefore, the method is extremely inefficient.

The subject matter of the present invention is to provide a method for efficiently obtaining base sequences that exist in spaces greatly different from those of naturally-existing base sequences, and also to provide genetic products to be obtained  
10 by expressing, as genes, the nucleic acid sequences that are obtained in that manner and that do not exist naturally.

The sequence space of a gene includes the full-length sequence thereof to be theoretically constituted by a combination of four bases, A, G, C and T. For example, a base sequence  
15 ce that codes for a protein composed of a number "n" of amino acids shall be constructed by selecting and sequencing any desired one of the four bases for a total of 3n-times, therefore including  $4^{3n}$  combinations. Accordingly, a protein composed of 100 amino acids shall include different base sequences of about  
20  $10^{18}$  types as so mentioned hereinabove.

In fact, there is no limitation for the number of amino acids that constitute proteins. Therefore, the sequencing spaces for proteins shall extend unlimitedly. During the steps of evolution of organisms, only a part of such sequencing spaces  
25 have been examined, and there is a great probability that some sequences coding for proteins which may have some extremely excellent functions could exist in the other great sequencing spaces. The protein engineering studies which have been and

are being made in many laboratories and institutes at present are essentially directed to the creation of novel proteins having functions superior to those of naturally-existing proteins, and one essential approach made therein to this purpose is to substitute amino acids in existing sequences, as so mentioned hereinabove. However, the amino acid substitution is nothing but the essential means that organisms have carried out during the steps of their evolution or, that is, such is the imitation of organisms and is to search only around the sequences that organisms already examined. In addition, there is a probability that the sequences thus obtained will be those that were already weeded out in the past.

We, the present inventors have considered that, in order to be greatly apart from the sequencing spaces that organisms already examined, if we carry out such matters that could not have been carried out by organisms, the purpose will be attained. We know that the division of a gene into several blocks followed by the change in the sequencing of the thus-divided blocks, if occurred in organisms, shall kill the organisms. Therefore, we have concluded that this method is suitable for our purpose. Having thus concluded, we, the present inventors have assiduously studied various matters relating to this method and, as a result, have found a method of forming a desired cohesive end or ends on a desired DNA. Utilizing this method, we have succeeded in a method of dividing a gene into several blocks and re-sequencing them into different sequences and also in a method of producing a molecule pool including such different base sequences existing in different

sequencing spaces, and thus have completed the present invention.

Accordingly, the present invention provides the following:

1) A DNA with a cohesive end comprising (a) a double-stranded DNA having the same sequence as that of a part of a gene, and (b) a single-stranded DNA having a base sequence that exists on said gene at the site not adjoining the part corresponding to said double-stranded DNA or a base sequence which said gene does not have, wherein the single-stranded DNA is linked to either one end of the double-stranded DNA to form a cohesive end.

2) A DNA with cohesive ends comprising (a) a double-stranded DNA having the same sequence as that of a part of a gene, (b) a first, single-stranded DNA having a base sequence that exists on said gene at the site not adjoining the part corresponding to said double-stranded DNA or a base sequence which said gene does not have, and (c) a second, single-stranded DNA having a base sequence that exists on said gene at the site adjoining the part corresponding to said double-stranded DNA, wherein the second, single-stranded DNA is linked to said double-stranded DNA at one end corresponding to said adjoining site, while the first, single-stranded DNA is linked thereto at the other end of the complementary strand opposite to said end, thereby forming cohesive ends.

3) The DNA with a cohesive end or cohesive ends according to the previous 1) or 2), wherein the single-stranded DNA has a length of 2 bases or more.



4) The DNA with a cohesive end or cohesive ends according to any one of the previous 1) to 3), wherein the cohesive end/ends is/are positioned at the 3'-terminal/terminals.

5) A method for producing a DNA with a cohesive end or  
5 cohesive ends, wherein a part of a DNA, as a template, and an oligonucleotide containing at least one ribonucleotide, as a primer, are subjected to DNA polymerase reaction to prepare a double-stranded DNA, then the ribonucleotide(s) is/are removed  
10 through enzymatic reaction or chemical reaction, and the nucleotide(s) remaining at the 5'-terminal(s) of the site(s) at which said ribonucleotide(s) existed are removed.

6) A method for producing the DNA with a cohesive end of the previous 1), comprising the following steps a) to d):

a) a step of linking (i) an oligonucleotide having the  
15 same base sequence as that of a part of a gene DNA to (ii) an oligonucleotide having a base sequence that exists on the gene at the site not adjoining the base sequence of (i) or a base sequence which the gene does not have, and containing at least one ribonucleotide, in such a manner that the oligonucleotide  
20 (ii) is positioned at the 5'-terminal of the oligonucleotide (i);

b) a step of preparing a double-stranded DNA through DNA polymerase reaction between a DNA containing the part corresponding to the oligonucleotide (i) in said a), as a template,  
25 plate, and the linked oligonucleotide as obtained in the previous step a), as a primer;

c) a step of removing the ribonucleotide from said double-stranded DNA through enzymatic reaction or chemical reaction; and

d) a step of removing the nucleotide remaining at the 5'-terminal of the site at which said ribonucleotide existed.

7) A method for producing the DNA with cohesive ends of the previous 2), comprising the following steps a) to d):

a) a step of linking (i) an oligonucleotide having the same base sequence as that of a part of a gene DNA to (ii) an oligonucleotide having a base sequence that exists on the gene at the site not adjoining the base sequence of (i) or a base sequence which the gene does not have, and containing at least one ribonucleotide, in such a manner that the oligonucleotide (ii) is positioned at the 5'-terminal of the oligonucleotide (i);

b) a step of preparing a double-stranded DNA through DNA polymerase reaction between a DNA containing the part corresponding to the oligonucleotide (i) in said a), as a template, and (i) the linked oligonucleotide as obtained in the previous step a) and (ii) an oligonucleotide which is a complementary strand of an oligonucleotide existing on the gene at the site separated from said oligonucleotide-corresponding part by at least 3 bases or more toward the 3'-terminal and which contains at least one ribonucleotide, as primers;

c) a step of removing the ribonucleotides from said double-stranded DNA through enzymatic reaction or chemical reaction; and

d) a step of removing the nucleotides remaining at the 5'-terminals of the sites at which said ribonucleotides existed.

8) A method for shuffling a DNA, comprising dividing a DNA into a plurality of DNA blocks each having a cohesive end or cohesive ends, followed by ligating them together into a sequence that is different from the sequence of the original, non-divided DNA.

9) A method for shuffling a DNA, comprising applying the method of any one of the previous 5) to 7) to various sites of a DNA, thereby dividing the DNA into a plurality of DNA blocks each having a cohesive end or cohesive ends, at least one block of which shall have a cohesive end that is complementary to the cohesive end of another block not having been directly adjacent to said one block on the original DNA, followed by ligating them together into a sequence that is different from the sequence of the original, non-divided DNA.

10) The shuffling method according to the previous 8) or 9), wherein the DNA is divided into 3 or more blocks.

11) The shuffling method according to any one of the previous 8) to 10), wherein the blocks are ligated together using a DNA ligase.

12) A DNA as shuffled according to the method of any one of the previous 8) to 11).

[0016]

13) The DNA according to the previous 12), wherein a gene coding for an enzymatic function or a control gene for the gene is shuffled.

14) The DNA according to the previous 13), wherein the gene is a gene that codes for any one of proteases, lipases, cellulases, amylases, catalases, xylanases, oxidases, dehydrogenases, oxygenases and reductases.

5 15) The DNA according to the previous 13) or 14), wherein the gene is one derived from prokaryotes.

16) The DNA according to the previous 15), wherein the gene is one derived from bacillus bacteria.

17) The DNA according to the previous 16), wherein the  
10 gene is a protease API21 gene.

18) A DNA pool containing plural kinds of DNAs having different structures that are obtained according to the shuffling method of any one of the previous 8 to 11).

19) The DNA pool according to the previous 18), which  
15 contains 10 or more kinds of DNAs.

20) A method for producing a DNA pool, comprising applying the method of any one of the previous 5) to 7) to various sites of a template DNA to thereby prepare a mixture of DNA blocks each having a cohesive end or cohesive ends that  
20 satisfies the following conditions, followed by ligating these into any desired sequences:

Condition 1: Each block has a double-stranded site having the same sequence as that of a part of the template DNA.

Condition 2: At least two of the blocks that constitute  
25 the block mixture further have, in addition to said double-stranded site, a single-stranded site (cohesive end) that is complementary to the cohesive end of blocks that are not directly adjacent to said blocks on the template DNA.

Condition 3: The block mixture contains at least two different blocks which are the same in the double-stranded site but are different only in the single-stranded site and which satisfy the condition 2.

5 21) The method for producing a DNA pool according to the previous 20), wherein the template DNA is a gene that codes for an enzymatic function or a control gene DNA for the gene.

22) The method for producing a DNA pool according to the previous 21), wherein the template DNA is a gene DNA that codes  
10 for any one of proteases, lipases, cellulases, amylases, catalases, xylanases, oxidases, dehydrogenases, oxygenases and reductases.

23) The method for producing a DNA pool according to the previous 22), wherein the template DNA is one derived from  
15 prokaryotes.

24) The method for producing a DNA pool according to the previous 23), wherein the template DNA is one derived from bacillus bacteria.

25) The method for producing a DNA pool according to the  
20 previous 24), wherein the template DNA is a protease API21 gene.

26) The method for producing a DNA pool according to any one of the previous 20) to 25), wherein the DNA blocks are ligated together using a DNA ligase.

25 27) A genetic product to be obtained by expressing the genetic information on DNA molecules that exist in the DNA pool of any one of the previous 18) to 26).

Now the present invention is described in detail hereinunder.

#### DNA with Cohesive End(s)

5       The present invention provides a DNA with any desired cohesive end or ends (herein referred to as "DNA with cohesive end(s)" unless otherwise specifically indicated). The cohesive end as referred to herein indicates a single-stranded site as protruded from the end of a double-stranded DNA. Such a  
10 cohesive end may be formed when a DNA is cleaved with a restriction enzyme such as EcoRI. In this case, however, the base sequence of the thus-formed cohesive end is defined, depending on the restriction enzyme used, and its length is generally composed of several bases or so. If a naturally-  
15 existing DNA is cleaved with a restriction enzyme, the sequence of the resulting double-stranded part of the DNA is also limited to the region as sandwiched between the restriction enzyme recognizing sites. As opposed to this, the DNA with cohesive end(s) of the present invention may have a structure in  
20 which a cohesive end or cohesive ends having a desired length and a desired sequence is/are added to the end or ends of a double-stranded DNA having a desired sequence.

As has been mentioned hereinabove, the sequence of the double-stranded part of the DNA with cohesive end(s) of the  
25 present invention is not specifically defined. For example, the sequence may be the same as that of a part of a gene. Though not also specifically defined, its length may be generally composed of 50 base pairs (bp) or more, preferably 45 bp

or more. The sequence of the cohesive end is not also specifically defined, but in order to prevent the self-ligation thereof in various reactions, it is preferable that the sequence does not form a stem structure. The "sequence to form  
5 a stem structure" as referred to herein includes, for example, AATT, which shall have just the same sequence as that of its complementary strand (TTAA). The length of the cohesive end may be generally 2 bp or more, preferably from 15 bp to 30 bp. If the cohesive end is too long, it may form a secondary structure whereby the intermolecular annealing will be difficult.  
10 However, if it is too short, its melting temperature ( $T_m$ ) is low and the annealing will be unstable.

The cohesive end may be linked to either the 3'-terminal or the 5'-terminal of the double-stranded DNA, but is preferably linked to the 3'-terminal thereof. The cohesive end may  
15 be linked to either only one terminal of the double-stranded DNA or the both terminals thereof.

#### Method for Producing DNA with Cohesive End(s)

20 The DNA with cohesive end(s) of the present invention can be produced typically according to a method comprising the following steps a) to d). The method mentioned below is directed to the production of a DNA with a cohesive end, which has a structure to be represented by a formula (2):

wherein  $\times$  and c are desired sequences; a and  $\bar{a}$  are sequences that are complementary to  $\times$  and c, respectively; and  $\omega$  is a sequence of a cohesive end, and which is based on a double-stranded DNA (template DNA) 5 having a structure to be represented by a formula (1):

(1)

wherein  $\times$ , c, a,  $\bar{a}$ , and  $\omega$  have the same meanings as above.

10 However, the present invention is not limited to only the production illustrated herein, but other DNAs with cohesive end(s) having other structures can also be produced in the same manner as below according to the present invention.

15 Step a)

(a-1) Preparation of Oligonucleotide:

First, the part that shall be selected as the double-stranded part of the intended DNA with a cohesive end is defined on a template DNA. An oligonucleotide, a, which is 20 complementary to its terminal,  $\times$ , and an oligonucleotide, c, having the same sequence as that of the other terminal, c, are prepared.  $\times$  and c each may have a sequence having a base length of from 15 to 30 bp or so.

On the other hand, prepared is an oligonucleotide, b, 25 which is complementary to the sequence to be prepared by removing one base (this is referred to as X) from the 5'-terminal of the sequence of the intended cohesive end,  $\omega$ .



The base sequence, ~~a~~, may be a part of the above-mentioned DNA or may be any other sequence that the DNA does not have.

These oligonucleotides, a, b and c, may be prepared by any methods. If their sequences are previously known, they can be synthesized, using a known DNA synthesizer.

(a-2) Preparation of Ribonucleotide-Containing Fragments:

Next, the oligonucleotides, a and b, are linked together via a ribonucleotide. This linkage can be attained by ordinary synthesizing methods. For example, it can be attained according to the process mentioned below.

First, a phosphoryl group is added to the 5'-terminal of the oligonucleotide, a, according to the reaction of the following formula (3):

15

(3)

wherein (P) is a phosphoryl group.

This reaction can be effected by the action of a polynucleotide kinase. ATP is used in an amount of from 2 to 10 times or so, by mol, relative to the oligonucleotide, a. The reaction temperature may be from 30 to 40°C or so. The reaction time may be from 10 minutes to 1 hour or so. Most suitably, the pH is from 7 to 9 or so. After the addition of the phosphoryl group thereto, the oligonucleotide is represented by a'.

[0024]

On the other hand, a ribonucleotide is added to the 3'-terminal of the oligonucleotide, b, according to the reaction of the following formula (4):

(4)

wherein X is any one of ATP, GTP, CTP and UTP; (rX) is a ribonucleotide.

This reaction can be effected by the action of, for example, a terminal deoxynucleotidyl transferase. For the nucleoside triphosphate (XTP) to be used herein, is selected a ribonucleotide that corresponds to the base X in the previous step (a-1). The nucleoside triphosphate is used in an amount of from 2 to 10 times, by mol, relative to the oligonucleotide, 10 b. The reaction temperature may be from 30 to 40°C or so. The reaction time may be from 30 minutes to 2 hours or so. After the addition thereto, the oligonucleotide is represented by b'. The sequence of b' is complementary to the sequence,  $a'$ .

The thus-obtained oligonucleotides, a' and b', are mixed, 15 whereby the 5'-terminal (phosphoryl group) of a' is bonded to the 3'-terminal (hydroxyl group) of the ribonucleotide of b', according to the reaction of the following formula (5):

(5).

20 This reaction can be effected by the action of an RNA ligase in the presence of ATP and divalent metal ions (Japanese Patent Application Laid-Open No. 5-292967). Divalent metal ions useful in this reaction include, for example, magnesium ions and manganese ions, but preferred are magnesium ions. As 25 the ligase, employable is an RNA ligase. The RNA ligase is an enzyme to catalyze the ligation of the hydroxyl group at the 3'-terminal and the phosphoryl group at the 5'-terminal, and this also efficiently catalyzes the ligation of a

polydeoxyribonucleotide having a ribonucleotide only at its 3'-terminal and a polydeoxyribonucleotide with a 5'-terminal phosphoryl group. Preferably used is a T4 RNA ligase. The reaction is generally effected in a buffer, at a pH of from 7 to 9 and at a temperature of from 10 to 40°C, over a period of from 30 to 180 minutes. For example, the oligonucleotides may be reacted in a solution comprising 50 mM Tris-HCl (pH 8.0), 20 mM MgCl<sub>2</sub>, 0.1 mM ATP, 10 mg/liter BSA, 1 mM hexaammine cobalt chloride (HCC) and 25 % polyethylene glycol 6000, at 25°C for 60 minutes or longer.

Step b)

Using the DNA containing the sequence,  $\times$ , as prepared in the previous step (a-1), as a template, and using the linked oligonucleotide, b'-a', as prepared in the previous step (a-2), as a primer, prepared is a double-stranded DNA through DNA polymerase reaction. In general, a double-stranded DNA containing the sequence,  $\times$ , and a sequence,  $\mathfrak{B}$ , on their strands is subjected to thermal or alkaline denaturation to give single-stranded DNAs, which are added to the primer of b'-a' and subjected to PCR with the oligonucleotide, c, as prepared in the previous step (a-1). The primer annealing condition and the polymerase reaction condition to be employed herein may be the same as those in ordinary polymerase reaction. The DNA polymerase to be employed herein may be any and every enzyme that can catalyze the DNA chain-extending reaction, which includes, for example, Taq polymerase, Klenow fragment, DNA polymerase I, etc. As a result of this reaction, obtained is a

double-stranded DNA with blunt ends, which is represented by the a formula (6):

(6).

Step c)

5       Next, the ribonucleotide is removed from the double-stranded DNA through enzymatic reaction or chemical reaction. One example of useful enzymes is a ribonuclease. The reaction is generally effected at a pH of from 6 to 8 or so, at from 30 to 70°C or so, over a period of from 10 to 60 minutes or so.  
10 As non-enzymatic chemicals usable herein, mentioned are sodium hydroxide and the like. As a result of this reaction, obtained is a partly-discontinuous, double-stranded DNA of the following formula (7), in which the part corresponding to the above-mentioned base, X, has been deleted.

15

(7).

Step d)

After the above step, the nucleotide existing at the 5'-terminal of the above-mentioned deletion is removed. To remove  
20 this nucleotide, for example, the double-stranded DNA, from which the ribonucleotide has been removed in the previous step c), is heated at from 50 to 90°C or so. The polynucleotide that has been separated from the strand through this reaction can be removed, using, for example, a span column or the like.  
25 Thus is obtained the double-stranded DNA with a cohesive end of the above-mentioned formula (2).

In the process mentioned above, obtained is a double-stranded DNA with a cohesive end only at its one 3'-terminal.

In the same manner as this, also obtainable is a double-stranded DNA with cohesive ends at the both 3'-terminals.

In the above-mentioned process, a desired sequence,  $\mathcal{S}$ , which does not adjoin the sequence,  $\mathcal{X}$ , in the template DNA was introduced into the DNA to form the cohesive end. Apart from this, it is also possible to introduce thereinto an additional oligonucleotide that adjoin the sequence in the template DNA to form another cohesive end. For example, in the embodiment mentioned above, an oligonucleotide,  $c'$ , which is different from the oligonucleotide,  $c$ , in that its 3'-terminal deoxyribonucleotide is substituted with a ribonucleotide, may be used as the primer in place of the oligonucleotide,  $c$ , to prepare a double-stranded DNA with two cohesive ends of a formula (8):

15

(8).

#### Method of Shuffling DNA

The present invention also provides a method of shuffling a DNA, which is characterized by using DNAs with cohesive end(s). The terminology "shuffling" as referred to herein indicates the operation of dividing a DNA into plural blocks followed by re-sequencing them into a desired, different sequence.

For example, where one DNA has a sequence composed of a number,  $n$ , of blocks, as represented by a formula (9):

25

$$A - a_1 - a_2 - \dots - a_n - B \quad (9)$$

wherein the starting end A and/or the terminal end B may be omitted,

this may be shuffled according to the present invention to give a different DNA to be represented by a formula (10):

$$A - a_1' - a_2' - \dots - a_x - B \quad (10)$$

wherein  $a_1'$ ,  $a_2'$ , . . . ,  $a_x$  are blocks that are  
 5 independently selected from the group of  $a_1$ ,  $a_2$ , . . . ,  
 $a_n$ ; and the total number of the blocks  $a_1'$ ,  $a_2'$ , . . . ,  
 $a_x$  may not be the same as the total number of the blocks  
 $a_1$ ,  $a_2$ , . . . ,  $a_n$ .

The principle of the DNA shuffling of the present  
 10 invention which utilizes DNAs with cohesive end(s) is graphically illustrated in Fig. 1. In Fig. 1, the DNA is shuffled at the intermediate part,  $p_1 - p_2 - p_3$  (the uppermost row) into  $p_3 - p_1 - p_2$  (the lowermost row), without changing the both ends  $p_A$  and  $p_B$ . This shuffling operation is useful as a method  
 15 for obtaining gene sequences that have not heretofore existed naturally, without changing the sequences of the promoter and the terminator.

Concretely, the above-mentioned method of preparing DNAs with cohesive end(s) is applied first to the parts  $p_A$ ,  $p_1$ ,  $p_2$ ,  
 20  $p_3$  and  $p_B$  constituting the template DNA, to thereby prepare DNA blocks,  $a_1$ ,  $a_2$  and  $a_3$ , each having the structure with two cohesive ends (formula (8)), and DNA blocks, A and B, each having the structure with one cohesive end (formula (2)). The cohesive ends,  $a_A$ ,  $a_{1f}$ ,  $a_{2f}$  and  $a_{3f}$ , are formed by removing the  
 25 corresponding complementary strand from the blocks,  $p_A$ ,  $p_1$ ,  $p_2$  and  $p_3$ , respectively.

The cohesive ends,  $a_{1r}$ ,  $a_{2r}$ ,  $a_{3r}$  and  $a_B$ , are designed according to the desired sequence to be prepared after the shuffling. In the embodiment of Fig. 1, the end,  $a_{1r}$  is designed to be a complementary strand to the end,  $a_{3f}$ , and the  
5 block,  $a_1$  is ligated to the block  $a_3$  after the shuffling. The ligation is conducted, using a DNA ligase in the presence of ATP. The type of the DNA ligase to be employed herein is not specifically defined. In this embodiment, since the single-stranded part of each cohesive end is long, it is unnecessary  
10 to employ the ordinary reaction at 16°C, but a thermophilic DNA ligase is advantageously employed.

In the embodiment of Fig. 1,  $a_{2r}$ ,  $a_{3r}$  and  $a_B$  are designed to be the complementary strands to  $a_{1f}$ ,  $a_A$  and  $a_{2f}$ , respectively, in the same manner as above. As a result of the shuff-  
15 ling, a sequence having a structure of A -  $a_3$  -  $a_1$  -  $a_2$  - B is finally obtained. This is seemingly the same as the re-sequenced order of  $p_A$  -  $p_3$  -  $p_1$  -  $p_2$  -  $p_B$  to be obtained by dividing the original DNA into the constitutive blocks  $p_1$ ,  $p_2$ ,  $p_3$ ,  $p_A$  and  $p_B$ , followed by re-sequencing these into a different  
20 sequence.

Any other desired sequences can be realized in the same manner as above. If the block, A or B, is made to have two cohesive ends, while the other blocks are made to have one cohesive end, it is possible to obtain still different sequences  
25 through shuffling where the latter blocks with one cohesive end are positioned at the terminals.

In the shuffling of the invention, it is also possible to introduce foreign DNA block(s) with cohesive end(s), which are

not in the original gene, into the gene DNA. For example, it is possible to shuffle two or more gene DNAs. In this case, the terminal of one gene, for example, the block A in the above-mentioned embodiment, may be processed into a DNA block with two cohesive ends, if desired.

The blocks, which are the units to be shuffled, are oligonucleotides or polynucleotides composed of 2 or more nucleotides (hereinafter referred to as "oligonucleotides"). In general, these are preferably composed of 30 or more nucleotide units, more preferably 45 or more nucleotide units. The uppermost limit of the block length is not specifically defined, provided that the block length is shorter than the length of one gene. If, however, the block length is too large, the re-sequenced DNA to be obtained by the shuffling shall have many non-mutated base sequence parts. Therefore, in general, the block length is preferably within the range of from 10 to 35 % of the length of a gene.

Where the gene to be shuffled is a gene that codes for a protein, it is desirable that the gene blocks, oligonucleotides have the same reading frame before and after the division. Namely, the gene blocks to be shuffled are desirably so designed that they are translated to always give the corresponding amino acid sequences, irrespective of their relative positions in the shuffled sequence. For this, the double-stranded parts and the cohesive ends shall be selected for their codon units in accordance with the reading frame of the gene DNA to be shuffled.



Needless-to-say, it is unnecessary to conduct the division into segment blocks with genetic meanings. Namely, it is unnecessary to conduct the division of the gene DNA into the constitutive exons or segment blocks that correspond to the domains or modules of the protein which the gene DNA codes for. There is a probability that the shuffling at such sites would have been examined in the natural world in the past. In order to obtain base sequences that have not heretofore been examined in the natural world, it is desirable that the division of the gene DNA is effected inside the constitutive exons or at the sites corresponding to the inside of the domains or modules of the protein which the gene DNA codes for.

Employing such means, therefore, it is possible to obtain proteins which have different structures as a whole from those of natural proteins but which partly contain amino acid sequences that have been confirmed to be useful in the natural world. Accordingly, the probability of obtaining useful proteins by such means is enlarged, as compared with the means of synthesizing proteins totally at random.

The kind of the gene to be shuffled according to the present invention is not specifically defined. Employable herein is any and every gene that is composed of polynucleotide chains and contains a coding region necessary for expressing a protein or RNA. The nucleotide unit may contain any molecule of deoxyribonucleotides or ribonucleotides. For the purpose of finding out useful base sequences, preferred are genes coding for proteins, especially enzymes, or control genes for enzymatic functions. Examples of such enzymes include proteases, lipa-

s s, cellulases, amylases, catalases, xylanases, oxidases, dehydrogenases, oxygenases and reductases.

The kind of the gene to which the present invention is directed is not specifically defined but shall be such that, 5 when it is introduced into a suitable host, the host can produce the genetic product through expression of the gene. As examples, referred to are genes as cloned from living organisms, artificially synthesized genes, and even genes as cloned from living organisms and artificially mutated. For the 10 genes derived from living organisms, employable are prokaryotes with definite enzyme producibility. As examples of such prokaryotes, mentioned are bacillus bacteria. One example of the genes derived from such bacteria is a protease API21 gene derived from *Bacillus* NKS-21 (FERM BP-93-1) (Japanese Patent 15 Application Laid-Open No. 5-91876, Sequence Number 1).

#### DNA Pool

The present invention also provides a DNA pool to be obtained according to the above-mentioned shuffling method. 20 The "DNA pool" as referred to herein means a high-density mixture of two or more DNAs. The DNA pool of the present invention can contain a particular number or more, for example, 10 or more different DNA molecules having different structures. It is desirable that, when the mixture, DNA pool is directly 25 used in biochemical operation or reaction, it is in such a form that all the plural nucleic acid components constituting it can be reacted. However, the form of the mixture, DNA pool is not

specifically defined, and the DNA pool may be either in solution or dry mixture.

To produce the DNA pool, for example, a plurality of cohesive ends for each block are prepared in the above-mentioned shuffling process. Referring to the embodiment of Fig. 1, for example, when, for the cohesive end  $a_{1r}$  of the block  $a_1$ , complementary strands to the other cohesive ends,  $a_A$  and  $a_{2f}$ , are prepared in addition to the complementary strand to  $a_{3f}$ , then DNAs of  $A - a_1 - a_2 - B$  and  $A - a_1 - a_2 - a_1$  can be obtained. If a complementary strand to the other cohesive end  $a_{1f}$  of  $a_1$  is added, it is also possible to produce other DNAs comprising a series of the same blocks, such as  $A - a_1 - a_1 - a_1$ .

In the same manner, for the cohesive ends of  $a_2$  and  $a_3$ , if oligonucleotides that are complementary to the cohesive ends of the other blocks or complementary to the other cohesive end of themselves are added, other sequences comprising these can be produced.

In general, a DNA is divided into blocks of  $a_1, a_2, a_3, \dots, a_n$ . Then, each block is processed to have a cohesive end or cohesive ends according to the above-mentioned process. The cohesive ends are designed to be oligonucleotides that are complementary to the cohesive ends of the other blocks or are complementary to the other cohesive end of themselves. All or a part of the thus-obtained DNA blocks are mixed and ligated to each other, thereby producing a nucleic acid pool containing different nucleic acids composed of the blocks as differently sequenced at random.

**Expression of Genetic Information in Shuffled DNA r DNA Pool**

The thus-shuffled, single or mixed, double-stranded DNAs are blunted. The blunting may be omitted, if DNA blocks with one cohesive end are positioned at the ends of the shuffled, 5 double-stranded DNA. For example, the 5'-terminal of the sequence containing a DNA block with a predetermined promoter sequence, which is based on the direction of the promoter, is not made to have a cohesive end but is made to have a blunt end, while the 3'-terminal of the sequence containing a DNA block 10 with a predetermined terminator sequence, which is based on the direction of the terminator, is not made to have a cohesive end but is also made to have a blunt end. In that manner, it is possible to directly obtain a gene in which the blocks of the intended gene have been shuffled between the promoter and the 15 terminator, without blunting it. After this, the thus-shuffled DNA is inserted into a desired vector, preferably an expression vector such as pKK223-3, using a DNA ligase. the promoter sequence and the terminator sequence to be in the shuffled DNA are not limited to only one each, but a plurality of promoter 20 sequences and terminator sequences may be therein.

If desired, the polynucleotide blocks positioned at the both ends of the shuffled DNA may be designed to have suitable restriction enzyme recognizing sites. In this case, the DNA may be ligated to a suitable vector, using the defined re- 25 striction enzymes.

Next, the vector library thus produced in the manner mentioned above is introduced into a suitable host, in which the genetic information is expressed. Thus, the intended gene-

tic product with favorable properties and also the gene coding for it can be obtained. Any and every ordinary host can be used herein. Preferred examples of the host include cells of *E. coli*, bacillus bacteria, yeasts, and lactic acid bacteria.

5 If desired, in-vitro transcription systems and translation systems are also employable herein. In those cases, the genetic information can be expressed even when the gene is not ligated to a vector.

The "genetic information" as referred to herein indicates  
10 the information on a gene which is carried by a DNA and which is translated into a protein or is transcribed into RNA in a suitable living body by the DNA for itself or after having been ligated to any other DNA or RNA.

The genetic information that is expected to be expressed  
15 according to the method of the present invention is not specifically defined, but includes, for example, those on various genetic products, such as enzymes, antibodies, hormones receptor proteins and ribozymes, and those on various control functions of, for example, operators, promoters and attenua-  
20 tors.

#### Examples

Now, the present invention is described in detail hereinunder with reference to the following examples, which,  
25 however, are not intended to restrict the scope of the present invention.

Example 1: Production of DNA Pool

A nucleic acid pool was produced in accordance with the process mentioned below, based on the wild-type alkali protease (Japanese Patent Application-Laid Open No. 5-91876) as cloned  
5 from a protease API21 (*Bacillus* NKS-21; FERM BP-93-1) having a sequence of Sequence Number 1.

- (1) Step a): Preparation of Oligonucleotide Blocks for Primer  
(1-1) Synthesis of Oligonucleotide Blocks:

Using an automatic DNA synthesizer, Model 392  
10 (manufactured by Perkin Elmer Co.), synthesized were 14 oligonucleotides; oligo FW (Sequence Number 2), oligo RV (Sequence Number 3), oligo 1r (Sequence Number 4), oligo 1b (Sequence Number 5), oligo 1a (Sequence Number 6), oligo 2r (Sequence Number 7), oligo 2b (Sequence Number 8), oligo 2a  
15 (Sequence Number 9), oligo 3r (Sequence Number 10), oligo 3b (Sequence Number 11), oligo 3a (Sequence Number 12), oligo 4r (Sequence Number 13), oligo 4b (Sequence Number 14), oligo 4a (Sequence Number 15) and oligo A (Sequence Number 16). These are parts of the base sequence of API21 (Japanese Patent  
20 Application Laid-Open No. 5-91876) (including complementary strands), or oligonucleotides containing a part of the base sequence. However, the sequence of oligo 4a is to follow glutamine of Sequence Number 1 and, and this contains a termination codon of the gene. These oligonucleotides were so  
25 designed that they might be the best when the oligo A was overhung on the 3'-terminal of the amplified DNA in the experiment to follow hereinund r, using a Taq polymerase.

These oligonucleotides were synthesized in a DM trityl-on condition (that is, while the 5'-hydroxyl group was protected with dimethoxytrityl group), and purified through an OPC column. The reagents used herein were obtained from Perkin  
5 Elmer Co.

(1-2) Addition of Ribonucleotide to Blocks:

Next, 500 pmols of oligo 1r, 1 nmol of ATP and 10 units of terminal deoxynucleotidyl transferase were added to a standard solution comprising:

10 50 mM Tris-HCl buffer (pH 8.0)

10 mM MgCl<sub>2</sub>

5 mM DTT (dithiothreitol)

25 % PEG 6000

1 mM HCC (hexaammine cobalt chloride)

15 10 µg/ml BSA (bovine serum albumin),

to thereby make 10 µl in total. The resulting solution was left at 37°C for 1 hour.

Oligo 2r, oligo 3r, oligo 4r, oligo 1b, oligo 2b, oligo 3b and oligo 4b were processed in the same manner as above. These  
20 four polynucleotides thus formed are referred to as oligo 1r', oligo 2r', oligo 3r', oligo 4r', oligo 1b', oligo 2b', oligo 3b' and oligo 4b'.

(1-3) Phosphorylation:

500 pmols of oligo 1a, 1 nmol of ATP and 10 units of  
25 polynucleotide kinase were dissolved in the standard solution having the same composition as above to make 10 µl in total. The resulting solution was left at 37\_C for 1 hour. Oligo 2a, oligo 3a and oligo 4a were processed in the same manner as

above. These polynucleotides thus formed are referred to as oligo 1a', oligo 2a', oligo 3a' and oligo 4a'.

(1-4) Ligation of Oligonucleotide Blocks:

500 pmols of oligo 1a', 100 pmols of oligo 1b', 100 pmols  
5 of oligo 2b', 100 pmols of oligo 3b', 100 pmols of oligo 4b',  
which had been obtained in the above, as well as 1 nmol of ATP  
and 50 units of T4 RNA ligase were added to the same standard  
solution as that mentioned above to make 10  $\mu$ l in total, and  
these were reacted at 25°C for 4 hours.

10 The other combinations, oligo 2a' with oligo 1b', oligo  
2b', oligo 3b' and oligo 4b'; oligo 3a' with oligo 1b', oligo  
2b', oligo 3b' and oligo 4b'; and oligo 4a' with oligo 1b',  
oligo 2b', oligo 3b' and oligo 4b', were also reacted in the  
same manner as above. A mixture of the four polynucleotides  
15 thus formed as a result of this reaction, oligo 1a' ligated to  
oligo 1b', oligo 2b', oligo 3b' and oligo 4b', is referred to  
as oligo 1M; a mixture of the four polynucleotides, oligo 2a'  
ligated to oligo 1b', oligo 2b', oligo 3b' and oligo 4b', is  
referred to as oligo 2M; a mixture of the four polynucleotides,  
20 oligo 3a' ligated to oligo 1b', oligo 2b', oligo 3b' and oligo  
4b', is referred to as oligo 3M; and a mixture of the four  
polynucleotides, oligo 4a' ligated to oligo 1b', oligo 2b',  
oligo 3b' and oligo 4b', is referred to as oligo 4M.

(2) Steps b) to d): Formation of Gene Blocks

25 A template, plasmid pSDT812 (Japanese Patent Application  
Laid-Open No. 1-141596), which had been prepared by inserting,  
into the ClaI cleaving site of pHS396, the gene of the wild-  
type alkali protease as cloned from *Bacillus* NKS-21, was



subjected to PCR with primers, oligo 1M and oligo 2r'. The gene fragment as amplified through this reaction was treated with a ribonuclease, and then heated at 80\_C for 5 minutes, whereby the polynucleotide(s) positioned at the 5'-terminal of the ribonucleotide existing in the both strands or one strand was/were removed. As a result of this, prepared was a gene block with cohesive end(s). This gene block is referred to as block 1M.

The other four combinations, oligo 2M and oligo 3r', oligo 3M and oligo 4r', oligo 4M and oligo RV, and oligo FW and oligo 1r', were processed in the same manner as above. These blocks thus prepared are referred to as block 2M, block 3M, block B, and block F, respectively.

#### 15 Example 2: Shuffling

Block 1M, block 2M, block 3M, block B and block F of the same amount were blended and ligated together, using Pfu DNA ligase.

After the ligation, the reaction mixture was subjected to agarose gel electrophoresis, through which was collected the DNA fragment of about 1.5 kbp.

#### Example 3: Identification of Nucleic Acid Pool

The thus-collected DNA of about 1.5 kbp was digested with restriction enzymes, EcoRI and BamHI, then mixed with a plasmid, pHY300PLK (manufactured by Yakult Honsha Co.), which had been digested with restriction enzymes, EcoRI and BamHI and processed with an alkali phosphatase, and thus ligated

together, using a ligation kit (manufactured by Takara Shuzo Co.). Using the resulting DNA, cells of *E. coli* JM105 were transformed, from which were selected tetracycline-resistant transformants. From these transformants, plasmid DNAs were  
5 extracted, purified and analyzed according to ordinary methods. Thus were obtained 97 clones with a DNA of 1.5 kbp as inserted between the EcoRI and BamHI recognizing sites of pHY300PLK.

The base sequences of these DNAs thus obtained in the manner mentioned above were sequenced to analyze how block 1M,  
10 block 2M, block 3M, block F and block B were ligated in what order or, that is, how these were shuffled. As in the principle, block F was positioned at the first site while block B at the fifth site, and block 1M, block 2M and block 3M were shuffled between the two. Table 1 shows different types of  
15 shuffling, and the number of clones with each type.

Table 1

Type of Shuffling	Number of Clones	Type of Shuffling	Number of Clones
111	2	223	2
112	5	231	5
113	2	232	2
121	3	233	3
122	4	311	2
123	7	312	6
131	4	313	5
132	5	321	7
133	3	322	2
211	1	323	5
212	5	331	2
213	4	332	5
221	1	333	2
222	3		

As in the above, it has been confirmed that, if three  
5 blocks of one gene are shuffled according to the method of the  
present invention, a nucleic acid pool is obtained that covers  
all combinations of clones each containing the same or  
different three of these blocks.

10 Example 4: Screening of Genetic Products Obtained from Nucleic  
Acid Pool

The DNAs as produced in Example 3 were mixed. Using the  
resulting DNA mixture, cells of *Bacillus subtilis* UOT0999 were  
transformed. Tetracycline-resistant transformants were selec-  
15 ted. 300 transformants were replicated on a skim milk-con-  
taining medium plate, on which were found clear zones around  
the colonies of 12 transformants. Accordingly, it is under-

stood that the enzyme which the shuffled gene codes for can be selected depending on its activity. The base sequences of these 12 clones that formed the clear zones were analyzed, from which it was found that these were sequenced in the same order of blocks as in the wild-type enzyme.

#### Example 5: Detection of Genetic Products

From 10 clones (one clone forms halo, while 9 clones do not) as selected from the transformants that had obtained in Example 3, and also from the host, *Bacillus subtilis* UOT0999, full-length RNAs were prepared. These were processed with a ribonuclease-free deoxyribonuclease, in order to remove the influence of the plasmids on the hybridization to be effected later on. Next, using oligo 1r as the probe, these were subjected to Northern hybridization. As a result, all lanes corresponding to the RNA of the transformants gave detectable bands, but no band was detected on the lanes corresponding to the RNA of the host.

#### **Advantages of the Invention**

According to the present invention, provided is a double-stranded DNA molecule with any desired cohesive end or ends. Using this, it is possible to obtain various DNAs with various base sequences which are substantially apart from the naturally-existing base sequence spaces, and also a DNA pool of a mixture of such DNAs, through simple processes. Therefore, it is possible to obtain excellent genetic products, such as proteins and enzymes, which could not be obtained in

conventional methods and which were not examined by organisms in the past. In addition, according to the method of the present invention for producing a nucleic acid pool, it is possible to obtain a mixture of nucleic acids while optionally  
5 shuffling the constitutive blocks at random in the intermediate parts but fixing the terminal sequences to be predetermined, desired ones, and it is also possible to shuffle the constitutive blocks without changing the amino acid sequence which each block codes for. Therefore, as compared with a me-  
10 thod of producing a completely-randomized nucleic acid pool, there is a high possibility that useful genetic products can be produced according to the method of the present invention.

## Sequence Listing

Sequence Number: 1

Length of Sequence: 1122

5      Type of Sequence: Nucleic Acid

Number of Strands: Double-stranded

Topology: Linear

Kind of Sequence: Genomic DNA

Source: *Bacillus* NKS-21 (FERM BP-93-1)

10      Characteristics of Sequence:

Code Indicating Characteristics: Sig Peptide

Existing Site: 1 . . . 93

Method of Determining Characteristics: S

Code Indicating Characteristics: Mat Peptide

15      Existing Site: 104 . . . 1112

Method of Determining Characteristic: S

## Sequence:

ATG AAT CTT CAA AAA ATA GCC TCA GCG TTG AAG GTT AAG CAA TCG GCA48  
 Met Asn Leu Gln Lys Ile Ala Ser Ala Leu Lys Val Lys Gln Ser Ala  
 20      -100                      -95                      -90  
 TTG GTC AGC AGT TTA ACT ATT TTG TTT CTA ATC ATG CTA GTA GGT ACG96  
 Leu Val Ser Ser Leu Thr Ile Leu Phe Leu Ile Met Leu Val Gly Thr  
 -85                      -80                      -75  
 ACT AGT GCA AAT GGT GCG AAG CAA GAG TAC TTA ATT GGT TTC AAC TCA 144  
 25      Thr Ser Ala Asn Gly Ala Lys Gln Glu Tyr Leu Ile Gly Phe Asn Ser  
 -70                      -65                      -60                      -55  
 GAC AAG GCA AAA GGA CTT ATC CAA AAT GCA GGT GGA GAA ATT CAT CAT 192  
 Asp Lys Ala Lys Gly Leu Ile Gln Asn Ala Gly Gly Glu Ile His His  
 -50                      -45                      -40  
 30      GAA TAT ACA GAG TTT CCA GTT ATC TAT GCA GAG CTT CCA GAA GCA GCG 240  
 Glu Tyr Thr Glu Phe Pro Val Ile Tyr Ala Glu Leu Pro Glu Ala Ala  
 -35                      -30                      -25  
 GTA AGT GGA TTG AAA AAT AAT CCT CAT ATT GAT TTT ATT GAG GAA AAC 288  
 Val Ser Gly Leu Lys Asn Asn Pro His Ile Asp Phe Ile Glu Glu Asn  
 35      -20                      -15                      -10  
 GAA GAA GTT GAA ATT GCA CAG ACT GTT CCT TGG GGA ATC CCT TAT ATT 336  
 Glu Glu Val Glu Ile Ala Gln Thr Val Pro Trp Gly Ile Pro Tyr Ile  
 -5                      1                      5                      10

TAC TCG GAT GTT GTT CAT CGT CAA GGT TAC TTT GGG AAC GGA GTA AAA 384  
 Tyr Ser Asp Val Val His Arg Gln Gly Tyr Phe Gly Asn Gly Val Lys  
                             15                            20                            25  
 GTA GCA GTA CTT GAT ACA GGA GTG GCT CCT CAT CCT GAT TTA CAT ATT 432  
 5 Val Ala Val Leu Asp Thr Gly Val Ala Pro His Pro Asp Leu His Ile  
                             30                            35                            40  
 AGA GGA GGA GTA AGC TTT ATC TCT ACA GAA AAC ACT TAT GTG GAT TAT 480  
 Arg Gly Gly Val Ser Phe Ile Ser Thr Glu Asn Thr Tyr Val Asp Tyr  
                             45                            50                            55  
 10 AAT GGT CAC GGT ACT CAC GTA GCT GGT ACT GTA GCT GCC CTA AAC AAT 528  
 Asn Gly His Gly Thr His Val Ala Gly Thr Val Ala Ala Leu Asn Asn  
                             60                            65                            70  
 TCA TAT GGC GTA TTG GGA GTG GCT CCT GGA GCT GAA CTA TAT GCT GTT 576  
 Ser Tyr Gly Val Leu Gly Val Ala Pro Gly Ala Glu Leu Tyr Ala Val  
 15 75                            80                            85                            90  
 AAA GTT CTT GAT CGT AAC GGA AGC GGT TCG CAT GCA TCC ATT GCT CAA 624  
 Lys Val Leu Asp Arg Asn Gly Ser Gly Ser His Ala Ser Ile Ala Gln  
                             95                            100                            105  
 20 GGA ATT CAA TGG GCG ATG AAT AAT GGG ATG GAT ATT GCC AAC ATG AGT 672  
 Gly Ile Glu Trp Ala Met Asn Asn Gly Met Asp Ile Ala Asn Met Ser  
                             110                            115                            120  
 TTA GGA AGT CCT TCT GGG TCT ACA ACC CTG CAA TTA GCA GCA GAC CGC 720  
 Leu Gly Ser Pro Ser Gly Ser Thr Thr Leu Gln Leu Ala Ala Asp Arg  
                             125                            130                            135  
 25 GCT AGG AAT GCA GGT GTC TTA TTA ATT GGG GCG GCT GGA AAC TCA GGA 768  
 Ala Arg Asn Ala Gly Val Leu Leu Ile Gly Ala Ala Gly Asn Ser Gly  
                             140                            145                            150  
 CAA CAA GGC GGC TCG AAT AAC ATG GGC TAC CCA GCG CGC TAT GCA TCT 816  
 Gln Gln Gly Gly Ser Asn Asn Met Gly Tyr Pro Ala Arg Tyr Ala Ser  
 30 155                            160                            165                            170  
 GTC ATG GCT GTT GGA GCG GTG GAC CAA AAT GGA AAT AGA GCG AAC TTT 864  
 Val Met Ala Val Gly Ala Val Asp Gln Asn Gly Asn Arg Ala Asn Phe  
                             175                            180                            185  
 35 TCA AGC TAT GGA TCA GAA CTT GAG ATT ATG GCG CCT GGT GTC AAT ATT 912  
 Ser Ser Tyr Gly Ser Glu Leu Glu Ile Met Ala Pro Gly Val Asn Ile  
                             190                            195                            200  
 AAC AGT ACG TAT TTA AAT AAC GGA TAT CGC AGT TTA AAT GGT ACG TCA 960  
 Asn Ser Thr Tyr Leu Asn Asn Gly Tyr Arg Ser Leu Asn Gly Thr Ser  
                             205                            210                            215  
 40 ATG GCA TCT CCA CAT GTT GCT GGG GTA GCT GCA TTA GTT AAA CAA AAA1008  
 Met Ala Ser Pro His Val Ala Gly Val Ala Ala Leu Val Lys Gln Lys  
                             220                            225                            230  
 CAC CCT CAC TTA ACG GCG GCA CAA ATT CGT AAT CGT ATG AAT CAA ACA1056  
 His Pro His Leu Thr Ala Ala Gln Ile Arg Asn Arg Met Asn Gln Thr  
 45 235                            240                            245                            250  
 GCA ATT CCG CTT GGT AAC AGC ACG TAT TAT GGA AAT GGC TTA GTG GAT1104  
 Ala Ile Pro Leu Gly Asn Ser Thr Tyr Tyr Gly Asn Gly Leu Val Asp  
                             255                            260                            265  
 GCT GAG TAT GCG GCT CAA  
 50 Ala Glu Tyr Ala Ala Gln  
                             270                            272

## Sequence Number: 2

Length of Sequence: 20

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

5 Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

GATTTTAGAA TTCGCAGCGG

## 10. Sequence Number: 3

Length of Sequence: 25

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

15 Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

CCGGATTTCCT TAAAGCCCTG AATAA

## Sequence Number: 4

20 Length of Sequence: 17

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

25 Sequence:

ACAGTCTGTG CAATTTC

## Sequence Number: 5



Length of Sequence: 17

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

5 Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

GAAATTGCAC AGACTGT

Sequence Number: 6

10 Length of Sequence: 20

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

15 Sequence:

CCTTGGGGAA TCCCTTATAT

Sequence Number: 7

Length of Sequence: 17

20 Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

25 CCCAATACGC CATATGA

Sequence Number: 8

Length of Sequence: 17

Type of Sequence: Nucleic Acid

5 Number of Strand: Single-stranded

Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

TCATATGGCG TATTGGG

10

Sequence Number: 9

Length of Sequence: 20

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

15 Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

GTGGCTCCTG GAGCTGAACT

20 Sequence Number: 10

Length of Sequence: 16

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

25 Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

TCTGATCCAT AGCTTG

Sequence Number: 11

Length of Sequence: 16

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

5 Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

CAAGCTATGG ATCAGA

10 Sequence Number: 12

Length of Sequence: 20

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

15 Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

CTTGAGATTA TGGCGCCTGG

Sequence Number: 13

20 Length of Sequence: 17

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

25 Sequence:

TGAGCCGCAT ACTCAGC

Sequence Number: 14

Length of Sequence: 17

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

5 Kind of Sequence: Other Nucleic Acid, Synthetic DNA

Sequence:

GCTGAGTATG CGGCTCA

Sequence Number: 15

10 Length of Sequence: 20

Type of Sequence: Nucleic Acid

Number of Strand: Single-stranded

Topology: Linear

Kind of Sequence: Other Nucleic Acid, Synthetic DNA

15 Sequence:

TAATCCCTAA GGATGTACTG

#### Brief Description of the Drawing

Fig. 1 is a graphical view showing one embodiment of the method of  
20 the present invention for shuffling a DNA.

## INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13b/s)

<b>A.</b> The indications made below relate to the microorganism referred to in the description on page 24 , lines 12-15 to page      lines	
<b>B. IDENTIFICATION OF DEPOSIT</b> <div style="float: right;">Further deposits are identified on an additional sheet <input type="checkbox"/></div>	
Name of depositary institution  National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry	
Address of depositary institution (including postal code and country)  1-3 Higashi 1-chome, Tsukuba-shi, Ibaraki-ken, Japan	
Date of deposit  7 May 1985	Accession Number  FERM BP-93-1
<b>C. ADDITIONAL INDICATIONS</b> (leave blank if not applicable) This information is continued on an additional <input type="checkbox"/>	
Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
<b>D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE</b> (if the indications are not for all designated States)	
<b>E. SEPARATE FURNISHING OF INDICATIONS</b> (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

<div style="text-align: center; border-bottom: 1px solid black; margin-bottom: 5px;">For receiving Office use only</div> <div style="display: flex; align-items: center; margin-bottom: 10px;"> <input checked="" style="margin-right: 10px;" type="checkbox"/> <div>This sheet was received with the international application</div> </div> <div style="border-top: 1px solid black; padding-top: 5px;"> Authorized officer  <div style="text-align: center;">  Anne-Grethe Warrer-Madsen  Head Clerk </div> </div>	<div style="text-align: center; border-bottom: 1px solid black; margin-bottom: 5px;">For International Bureau use only</div> <div style="display: flex; align-items: center; margin-bottom: 10px;"> <input style="margin-right: 10px;" type="checkbox"/> <div>This sheet was received by the International Bureau</div> </div> <div style="border-top: 1px solid black; padding-top: 5px;"> Authorized officer </div>
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**CLAIMS**

1. A DNA with a cohesive end comprising (a) a double-stranded DNA having the same sequence as that of a part of a gene, and (b) a single-stranded DNA having a base sequence that  
5 exists on said gene at the site not adjoining the part corresponding to said double-stranded DNA or a base sequence which said gene does not have, wherein the single-stranded DNA is linked to either one end of the double-stranded DNA to form a cohesive end.
- 10 2. A DNA with cohesive ends comprising (a) a double-stranded DNA having the same sequence as that of a part of a gene, (b) a first, single-stranded DNA having a base sequence that exists on said gene at the site not adjoining the part corresponding to said double-stranded DNA or a base sequence  
15 which said gene does not have, and (c) a second, single-stranded DNA having a base sequence that exists on said gene at the site adjoining the part corresponding to said double-stranded DNA, wherein the second, single-stranded DNA is linked to said double-stranded DNA at one end corresponding to said  
20 adjoining site, while the first, single-stranded DNA is linked thereto at the other end of the complementary strand opposite to said end, thereby forming cohesive ends.
3. The DNA with a cohesive end or cohesive ends as claimed in claim 1 or 2, wherein the single-stranded DNA has a  
25 length of 2 bases or more.
4. The DNA with a cohesive end or cohesive ends as claimed in any one of claims 1 to 3, wherein the cohesive end/ends is/are positioned at the 3'-terminal/terminals.

5. A method for producing a DNA with a cohesive end or cohesive ends, wherein a part of a DNA, as a template, and an oligonucleotide containing at least one ribonucleotide, as a primer, are subjected to DNA polymerase reaction to prepare a double-stranded DNA, then the ribonucleotide(s) is/are removed through enzymatic reaction or chemical reaction, and the nucleotide(s) remaining at the 5'-terminal(s) of the site(s) at which said ribonucleotide(s) existed are removed.

6. A method for producing the DNA with a cohesive end as set forth in claim 1, comprising the following steps a) to d):

a) a step of linking (i) an oligonucleotide having the same base sequence as that of a part of a gene DNA to (ii) an oligonucleotide having a base sequence that exists on the gene at the site not adjoining the base sequence of (i) or a base sequence which the gene does not have, and containing at least one ribonucleotide, in such a manner that the oligonucleotide (ii) is positioned at the 5'-terminal of the oligonucleotide (i);

b) a step of preparing a double-stranded DNA through DNA polymerase reaction between a DNA containing the part corresponding to the oligonucleotide (i) in said a), as a template, and the linked oligonucleotide as obtained in the previous step a), as a primer;

c) a step of removing the ribonucleotide from said double-stranded DNA through enzymatic reaction or chemical reaction; and

d) a step of removing the nucleotide remaining at the 5'-terminal of the site at which said ribonucleotide existed.

7. A method for producing the DNA with cohesive ends as set forth in claim 2, comprising the following steps a) to d):

a) a step of linking (i) an oligonucleotide having the same base sequence as that of a part of a gene DNA to (ii) an  
5 oligonucleotide having a base sequence that exists on the gene at the site not adjoining the base sequence of (i) or a base sequence which the gene does not have, and containing at least one ribonucleotide, in such a manner that the oligonucleotide (ii) is positioned at the 5'-terminal of the oligonucleotide  
10 (i);

b) a step of preparing a double-stranded DNA through DNA polymerase reaction between a DNA containing the part corresponding to the oligonucleotide (i) in said a), as a template, and (i) the linked oligonucleotide as obtained in the  
15 previous step a) and (ii) an oligonucleotide which is a complementary strand of an oligonucleotide existing on the gene at the site separated from said oligonucleotide-corresponding part by at least 3 bases or more toward the 3'-terminal and which contains at least one ribonucleotide, as primers;

20 c) a step of removing the ribonucleotides from said double-stranded DNA through enzymatic reaction or chemical reaction; and

d) a step of removing the nucleotides remaining at the 5'-terminals of the sites at which said ribonucleotides existed.

25 8. A method for shuffling a DNA, comprising dividing a DNA into a plurality of DNA blocks each having a cohesive end or cohesive ends, followed by ligating them together into a



sequence that is different from the sequence of the original, non-divided DNA.

9. A method for shuffling a DNA, comprising applying the method as set forth in any one of claims 5 to 7 to various sites of a DNA, thereby dividing the DNA into a plurality of DNA blocks each having a cohesive end or cohesive ends, at least one block of which shall have a cohesive end that is complementary to the cohesive end of another block not having been directly adjacent to said one block on the original DNA, followed by ligating them together into a sequence that is different from the sequence of the original, non-divided DNA.

10. The shuffling method as claimed in claim 8 or 9, wherein the DNA is divided into 3 or more blocks.

11. The shuffling method as claimed in any one of claims 8 to 10, wherein the blocks are ligated together using a DNA ligase.

12. A DNA as shuffled according to the method as set forth in any one of claims 8 to 11.

13. The DNA as claimed in claim 12, wherein a gene coding for an enzymatic function or a control gene for the gene is shuffled.

14. The DNA as claimed in claim 13, wherein the gene is a gene that codes for any one of proteases, lipases, cellulases, amylases, catalases, xylanases, oxidases, dehydrogenases, oxygenases and reductases.

15. The DNA as claimed in claim 13 or 14, wherein the gene is one derived from prokaryotes.

16. The DNA as claimed in claim 15, wherein the gene is one derived from bacillus bacteria.

17. The DNA as claimed in claim 16, wherein the gene is a protease API21 gene.

5 18. A DNA pool containing plural kinds of DNAs having different structures that are obtained according to the shuffling method as set forth in any one of claims 8 to 11.

19. The DNA pool as claimed in claim 18, which contains 10 or more kinds of DNAs.

10 20. A method for producing a DNA pool, comprising applying the method as set forth in any one of claims 5 to 7 to various sites of a template DNA to thereby prepare a mixture of DNA blocks each having a cohesive end or cohesive ends that satisfies the following conditions, followed by ligating these  
15 into any desired sequences:

Condition 1: Each block has a double-stranded site having the same sequence as that of a part of the template DNA.

Condition 2: At least two of the blocks that constitute the block mixture further have, in addition to said double-  
20 stranded site, a single-stranded site (cohesive end) that is complementary to the cohesive end of blocks that are not directly adjacent to said blocks on the template DNA.

Condition 3: The block mixture contains at least two different blocks which are the same in the double-stranded site  
25 but are different only in the single-stranded site and which satisfy the condition 2.

21. The method for producing a DNA pool as claimed in claim 20, wherein the template DNA is a gene that codes for an enzymatic function or a control gene DNA for the gene.

22. The method for producing a DNA pool as claimed in  
5 claim 21, wherein the template DNA is a gene DNA that codes for any one of proteases, lipases, cellulases, amylases, catalases, xylanases, oxidases, dehydrogenases, oxygenases and reductases.

23. The method for producing a DNA pool as claimed in claim 22, wherein the template DNA is one derived from  
10 prokaryotes.

24. The method for producing a DNA pool as claimed in claim 23, wherein the template DNA is one derived from bacillus bacteria.

25. The method for producing a DNA pool as claimed in  
15 claim 24, wherein the template DNA is a protease API21 gene.

26. The method for producing a DNA pool as claimed in any one of claims 20 to 25, wherein the DNA blocks are ligated together using a DNA ligase.

27. A genetic product to be obtained by expressing the  
20 genetic information on DNA molecules that exist in the DNA pool as set forth in any one of claims 18 to 26.

1/1

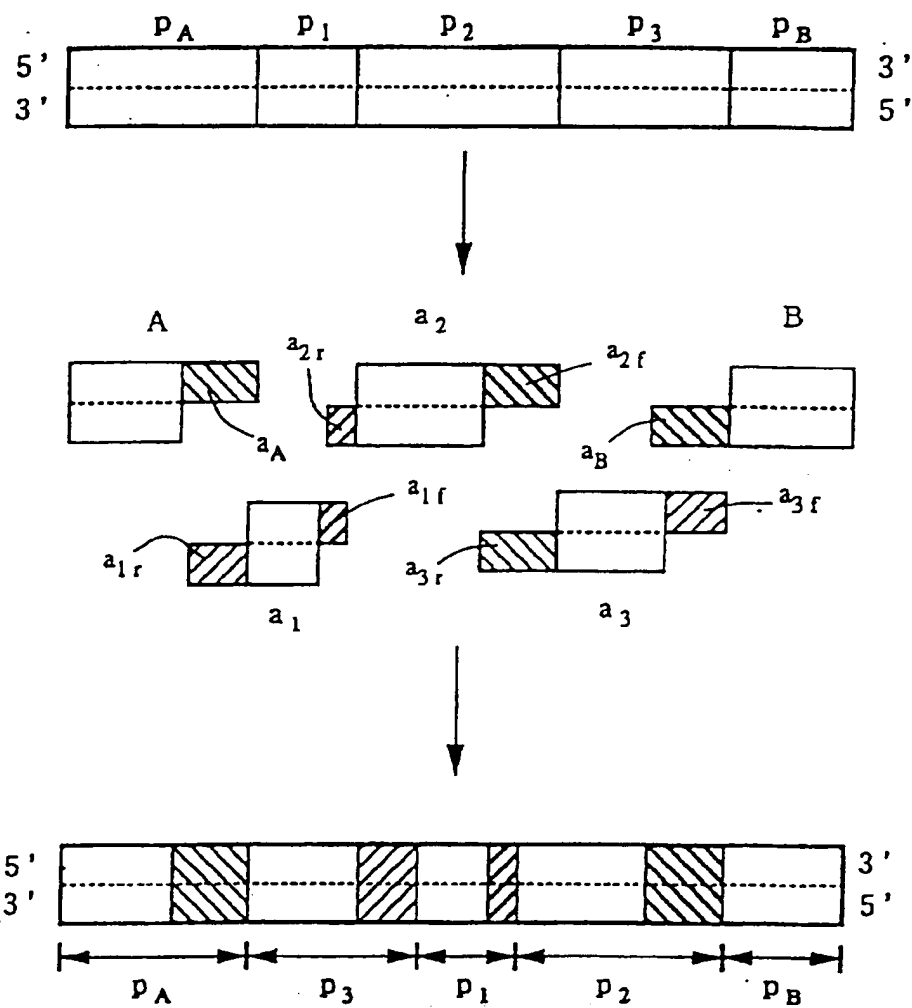


Fig. 1

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00317

## A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C12N 15/10, C12Q 1/68 // C12N 9/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C12N, C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, MEDLINE, DBA, BIOSIS, SCISEARCH

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemistry Letters, Volume 2, 1995, Koichi Nishigaki et al, "Restriction-Enzyme-Nondependent Recombination and Rearrangement of DNA (RRR)" page 131 --	1-27
X	WO 9107506 A1 (UNITED STATES OF AMERICA), 30 May 1991 (30.05.91), fig. 7 and the whole document --	1-4
X	WO 9517413 A1 (EVOTEC BIOSYSTEMS GMBH), 29 June 1995 (29.06.95), the whole document, see especially page 8, line 3-7, page 9, line 15-16, page 16, line 7-11 and claims --	8-19,27

☒ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

\* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

Date of mailing of the international search report

21 November 1997

01-12-1997

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# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00317

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>WO 9522625 A1 (AFFYMAX TECHNOLOGIES N.V.),  24 August 1995 (24.08.95), the whole document, see  especially page 44, line 34 - page 45, line 15</p> <p style="text-align: center;">--  -----</p>	8-19,27

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00317

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see next sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☒ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.  
☐ No protest accompanied the payment of additional search fees.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00317

According to PCT rule 13.2, an international application shall relate to one invention only or a group of inventions linked by one or more of the same or corresponding "special technical features", i.e. features that define a contribution which each of the inventions makes over the prior art.

Such a unifying link would be a DNA sequence with a cohesive end comprising a double stranded DNA sequence from a gene linked to at least one single stranded DNA not adjoining the double stranded DNA sequence in the gene. However such a DNA sequences are known in the art see e.g. WO9107506 or Nishigaki et.al in the search report. No other unifying special technical feature have been found.

The application is considered to comprise of the following inventions:

Invention 1, claims 1-4: DNA sequence with a cohesive end comprising a double stranded DNA sequence from a gene linked to at least one single stranded DNA not adjoining the double stranded DNA sequence in the gene.

Invention 2, claims 5-7 and 20-26, and related parts of claim 27: A method for producing DNA sequence with cohesive ends using ribonucleotides as a primer to create a double stranded DNA with polymerase reaction, whereafter the ribonucleotides are removed to create a cohesive end and a method for producing a DNA pool by applying the method.

Invention 3, claim 8-19 and related parts of claim 27: A method for shuffling a DNA and a DNA pool containing DNAs obtained by the method.

In spite of the non-unity all claims have been searched.



## INTERNATIONAL SEARCH REPORT

01/10/97

International application No.

PCT/DK 97/00317

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9107506 A1	30/05/91	AU 6886991 A	13/06/91
WO 9517413 A1	29/06/95	DE 4343591 A	22/06/95
WO 9522625 A1	24/08/95	AU 2971495 A	04/09/95
		CA 2182393 A	24/08/95
		CN 1145641 A	19/03/97
		EP 0752008 A	08/01/97
		US 5605793 A	25/02/97